SYNTHESIS AND FLUORESCENT PROPERTIES OF NEW HETEROBIFUNCTIONAL FLUORESCENT PROBES

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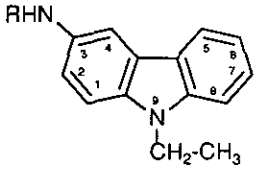
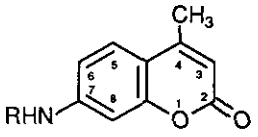
Abstract - Heterobifunctional fluorescent molecules possessing the same fluorescent properties as their monofunctional parent compounds are investigated. Two different functions of these probes do not alter their lasing properties and allow many potential applications in cellular biochemistry. This paper investigates two families of compounds derived from carbazole and coumarin. The synthesis and spectral properties of these probes are described.

3-Amino-9-ethylcarbazole (AEC) (1) and the 7-amino-4-methylcoumarin (AMC) (2) are two of the most current fluorescent reagents used in the synthesis and application of various fluorogenic substrates.[1,2]
They possess the basic requirements for use in fluorogenic enzyme substrates which have a relatively high quantum yield and a high shift of the emission spectrum of the free amine upon hydrolysis of the acyl derivative.[3, 4] Their amine function can react with carboxylic ends of short peptides such as enzyme susbtrates to form fluorogenic probes leading to a number of biological applications.[5-8]



1a: R = H (AEC)
1b: R = CH_3CO



2a: R = H (AMC)
2b: R = CH_3CO

For example, peptide derivatives in which a fluorescent amine is released upon proteolysis are currently the best tools for the assay of extremely low protease activities[3] and these compounds can be used in histoenzymatic methods in order to visualize *in situ* certain proteases and peptidases.[9] However, use of fluorogenic reagents is limited due to the relatively low molecular weight of the complex. This causes a diffusion of the released probe which does not remain at the target enzyme and a drop in the *in situ* detection sensitivity.

The use of heterobifunctional fluorescent compounds bearing an amino group and a carboxylic function can resolve this problem. The amine function can react with the carboxylic ends of peptides and the carboxylic group of the probe can form bridges with side chains of polymers or lateral chains of aminoacids in carrier proteins.

The fixation of certain enzyme substrates on a molecular support (such as a polymer) considerably increases the molecular weight of the substrate thus allowing a concentration of the fluorescent entities released at the location of the protease. *In situ* detection sensitivity for peptidases and proteases is then improved because of increased fluorescence.

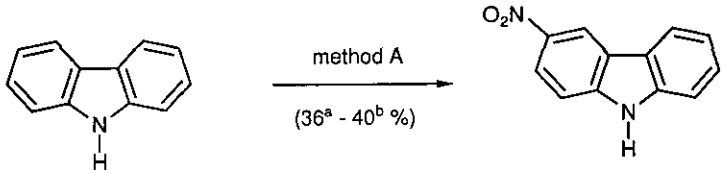
By studying various biological systems, we hope to develop a series of fluorescent reagents employing heterobifunctional molecules as key fluorophores. These products, derived from 3-amino-9-ethylcarbazole (**1a**) and 7-amino-4-methylcoumarin (**2a**), should conserve the fluorescent properties of their parent compounds. This paper describes their synthesis and spectral properties which are compared with those of **1a** and **2a**.

RESULTS AND DISCUSSION

3-Aminocarbazolyl derivatives.

For the synthesis of the carbazolyl derivatives, two methods were tested. In method A, we had expected to obtain the 3-nitrocarbazole (**4**) without protection of the carbazole nitrogen[10] with a better yield (Scheme 1).

Scheme 1

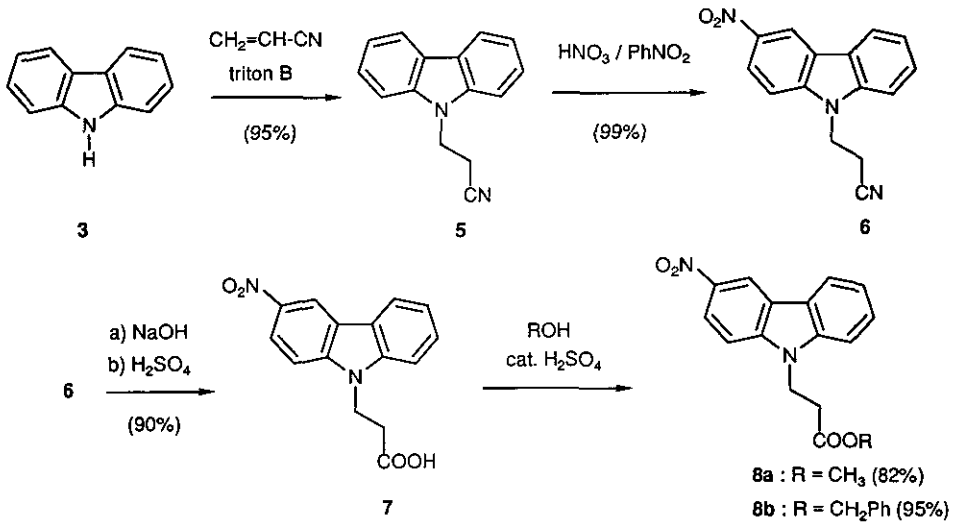


3 **4**

[a] : 1) $NaNO_2$, HCl / HNO_3, $PhNO_2$; 2) 10% KOH in ethanol
[b] : 1) $NaNO_2$, CH_3COOH; 2) HNO_3 / CH_3COOH; 3) 10% KOH in ethanol

However, these procedures were not satisfactory and, in method B, protecting the carbazole nitrogen before nitration is necessary (Scheme 2).

Scheme 2: method B



3 **5** **6**

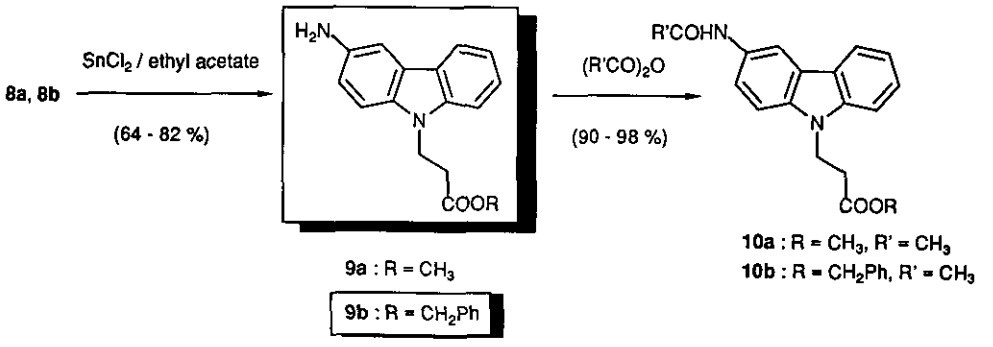
7

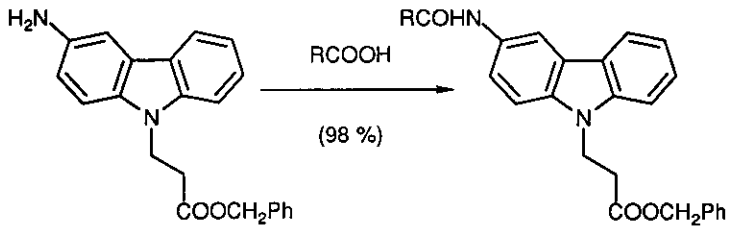
8a : R = CH_3 (82%)
8b : R = CH_2Ph (95%)

Substitution occurs with a Michael condensation using **3** and acrylonitrile in the presence of a base. The use of sodium ethoxide did not provide the desired product with a good yield (30%). In contrast, the procedure described by Whitmore[11] using benzyltrimethylammonium hydroxide (triton B) provided an excellent yield (95%). The nitration of the 9-cyanoethylcarbazole (**5**) in position 3 was easily obtained by treatment with nitric acid in the presence of nitrobenzene[12] with a yield of 99%. 3-(3-Nitro-9-carbazolyl)propanenitrile (**6**) was then saponified with a good yield (90%) by 10% sodium hydroxide.[13] 3-(3-Nitro-9-carbazolyl)propanoic acid (**7**) was esterified by a classical method[14] using methanol or benzyl alcohol with a catalytic amount of sulfuric acid. Methyl and benzyl 3-(3-nitro-9-carbazolyl)propionate (**8a**, **8b**) were obtained with 82 and 95% yields respectively. The 3-amino derivatives (**9a**, **9b**) were not easily obtained and several methods were tried.[15-17] Many of them did not produce the desired compound but some variations of a procedure using hydrazine / Raney nickel[18] were applied in several solvents and provided **9b** with yields between 30 and 80%. The best method, derived from a method described by Bellamy *et al.*,[19] provided the desired product with good yields (64-82%).

The *N*-acylated products (**10a**) and (**10b**) were obtained from **9b** by classical methods.[20] The treatment of **9a** and **9b** by acetic anhydride in pyridine provided the compounds (**10a**) and (**10b**) with a good yield (90-98%). The stirring of **9b** with *N*-protected L-alanine in the presence of hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCCI) provided **10c** with a yield of 90% (Scheme 3).

Scheme 3

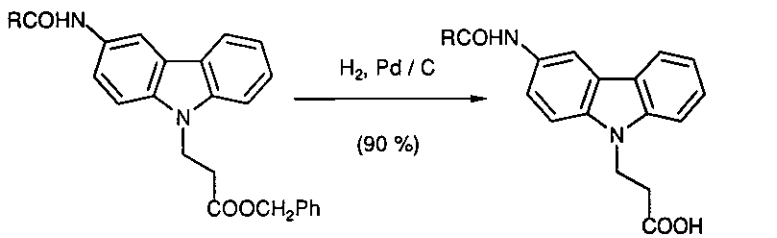




9b **10c** : R = tBOC-NHCH(CH_3), tBOC-ala

These compounds were easily debenzylated with hydrogen in the presence of Pd/C as catalyst. This method provided **11a** and **11b** with a good yield of 90% (Scheme 4).

Scheme 4



10b : R = CH_3 **11a** : R = CH_3

10c : R = tBOC-NHCH(CH_3) **11b** : R = tBOC-NHCH(CH_3)

The carbazole derivatives obtained were used as fluorescent model compounds in spectral studies.

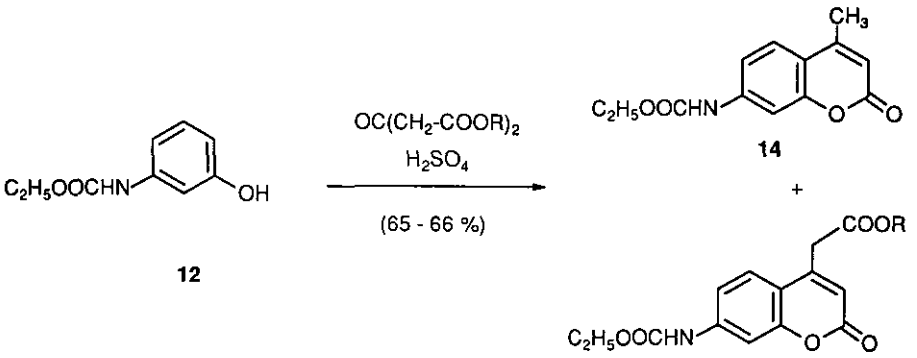
7-Aminocoumarin derivatives.

The key starting compound of a series of azidocoumarins was the ethyl 7-aminocoumarin-4-acetate (**13b**) wich was described by Kanaoka *et al.*[21] This compound was obtained with a yield of 41% after stirring 3-ethoxycarbonylaminophenol (**12**) and diethyl acetonedicarboxylate in 75% sulfuric acid and the precipitation of the product with ice water. In our method dimethyl acetonedicarboxylate and **12** was also used as starting products by stirring without solvent but with a catalytic amount of sulfuric acid. After thirty minutes a white precipitate was formed and methyl ester (**13a**) was crystallized in ethanol with a yield of 66%. The ethyl ester (**13b**) was synthesized by our method with a 65% yield.

It was observed that the reaction did not occur with the free aminophenol and that the use of a

carbethoxyaminophenol was necessary for this synthesis. In our method, 7-ethoxycarbonylamino-4-methylcoumarin (**14**) was probably formed after the partial decarboxylation of the dimethyl or diethylacetonedicarboxylate in the presence of sulfuric acid. The products obtained could be separated by flash chromatography for the methyl ester (**13a**) or by hplc for the ethyl ester (**13b**) (Scheme 5).

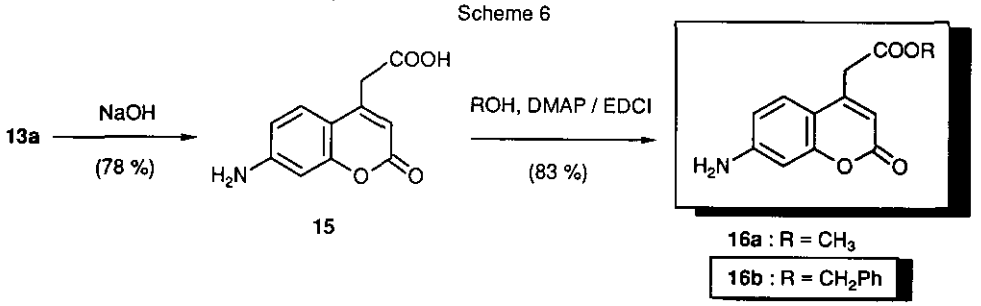
Scheme 5



13a : R = CH_3
13b : R = C_2H_5

Compound (**13a**) was then saponified with 10 equivalents of sodium hydroxide in water. 7-Aminocoumarin-4-acetic acid (**15**) was obtained in 78% yield (instead of the 51.8% yield previously published[21]). The acid function of **15** could easily be esterified to provide **16a** (81%) and **16b** (83%) by a method described for the protection of amino acids[22] (this method was successfully used in the ester formation of coumarins bearing a carboxylic acid function[23]) (Scheme 6).

Scheme 6

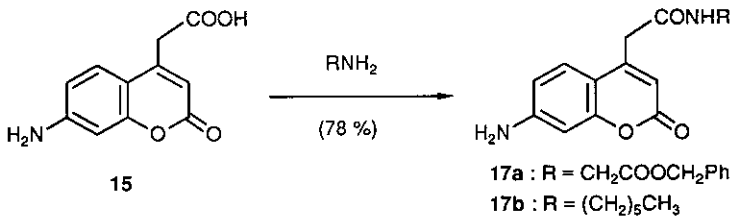


16a : R = CH_3
16b : R = CH_2Ph

In preliminary studies on the reactivity of the coumarin amino acid (**15**), we observed that the coumarin acid function could easily react in classical coupling reactions with free amine functions. By contrast, it was

impossible to protect the coumarin amine function by classical t-butoxycarbamates using methods improved with other amino acids.[24, 25] The reactivity of this heterocyclic amine function appeared to be so poor that the coumarin acid function could react with another free amine without any competition between the two amines in solution. This was confirmed in the synthesis of compounds (**17a**) and (**17b**) obtained with a yield of 78% after treatment of **15** with a *C*-protected benzylglycine or with hexylamine in presence of hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCCI) (Scheme 7).

Scheme 7



Products (**18a**, **18b**, and **19**) were easily provided by acylation of **16a**, **16b**, **17a**, respectively (Scheme 8). Compound (**16b**) and its acyl derivative (**18b**) served as model compounds in the spectral studies and the spectral data were compared with those of the carbazole benzyl ester derivative.

Scheme 8

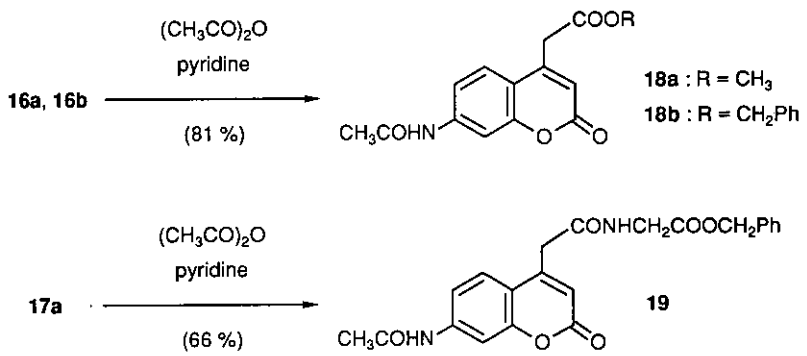


Table : Spectral data of some compounds (in ethanol).

Compounds	UV λ max (nm)	Log ε	Fluorescence λ max (nm)	Quantum yield[a]
1a[b]	360	3.39	460	-
1b	304	3.34	390	-
2a	354	4.07	435	0.88[c]
2b[d]	328	3.60	385	0.23
9a	363	3.32	440	0.30
9b	363	3.39	459	0.25
10b	341	3.35	399	0.21
10c	341	3.41	398	0.17
13a	331	4.10	416	0.78
15	354	3.95	454	1.19
16a	362	4.08	462	0.86[e]
16b	362	4.15	460	0.85
17a	359	3.70	462	1.49
18a	332	4.16	394	0.43
18b	330	3.94	390	0.42
19	330	4.09	415	0.53

[a] Calculated by a method previously described,[26, 27] based on a value of 0.55 for quinine sulfate.[28]

[b] Spectral data previously published.[3]

[c] Data previously published by Still *et al.*[29]

[d] Obtained by treatment of **2a** with acetic anhydride in the presence of pyridine.

[e] In accordance with the value of 0.83 published by Kanaoka *et al.*[21]

Fluorescent properties.

Our studies show that the two heterobifunctional amines and their acyl derivatives mimic perfectly the fluorescent properties of **1a** and **2a** (Table). They have an excellent bathochromic effect and a sufficiently large overlapping of the absorption and emission spectra. We observed a good shift of the emission spectrum of the free amines in comparison with their acyl derivatives. This explains why at the wavelength emission of the free amine, the fluorescence of the *N*-acylated compounds can be considered insignificant. This is one of the basic requirements for use in fluorogenic enzyme substrates. The spectral data of compounds (**9b, 10b** and **10c**) show that the synthesized compounds possess the same characteristics as their parent compounds (**1a**) and (**1b**).

As expected, our study of heterobifunctional coumarins bearing a carboxylic function directly on the coumarin ring,[23] the presence of one carbon between the coumarin ring and the carboxylic function was sufficient enough to reduce the influence of the acid function thus inducing good lasing properties of these compounds.

Comparison of the two predominant products (**9b**) and (**16b**) showed that the absorption and the emission spectra of the two types of probes are nearly the same, but their quantum yields are very different. The coumarin derivatives appear to be the best products for detection using "blue" fluorescence in appearance.

The spectral data of **16a, 16b** and **17a** do not indicate any influence of the acid function substituent on fluorescence. The molecules synthesized present the same fluorescent properties as the 7-amino-4-methylcoumarin (**2a**) which is the most useful commercial probe.

The existence of two different functions on these probes and their attractive fluorescent properties suggests a number of applications, and some of these dyes will be used to study certain cellular and biological systems, especially *in situ* detection of enzymes.[9]

EXPERIMENTAL

Melting points are uncorrected. The ir spectra were obtained by using a Perkin-Elmer 196 and the ^{1}H nmr spectra were recorded at 300 MHz on a Bruker AM 300WB spectrometer. Chemical shifts are reported in parts per million downfield from tetramethylsilane (δ units). Analytical thin layer chromatography (tlc) was performed on Merk 60F-254 silica gel plates. Preparative column chromatography was performed by using Merck silica gel 60F (70-230 mesh). Mass spectra were measured on a Nermag R-10-10C spectrometer,

absorption spectra were run on a Hitachi U-2000 spectrophotometer and fluorescence spectra at room temperature on a Shimadzu RF-500 fluorimeter.

9-Cyanoethylcarbazole (**5**)

A solution of carbazole (**3**) (5 g, 20 mmol) in acrylonitrile (9.5 ml, 144 mmol) is cooled with stirring in an ice bath. *N*-benzyltrimethylammonium hydroxide (triton B, 0.06 ml, 0.33 mmol) is added and the reaction mixture is stirred for 20 min at 0°C. The pale yellow mass obtained is stirred for 1 h at room temperature and the precipitate formed is collected by filtration, washed and dried to provide 4.18 g (95%) of **5** as pale yellow cubes; mp: 153-155°C (from isopropanol); ir (KBr): 2240 (CN) cm^{-1}; ^1H nmr (DMSO-d_6) δ = 3.04 (t, J = 6.91 Hz, 2H, -CH_2CN), 4.73 (t, J = 6.91 Hz, 2H, NCH_2-), 7.23 (t, J = 7.70 Hz, 2H, $H_{arom.}$), 7.46 (t, J = 7.70 Hz, 2H, $H_{arom.}$), 7.71 (d, J = 7.70 Hz, 2H, $H_{arom.}$), 8.15 (d, J = 7.70 Hz, 2H, $H_{arom.}$); ms: (m/z) 221 (M^+). *Anal.* Calcd for $C_{15}H_{12}N_2$: C, 81.79; H, 5.49; N, 12.72. Found: C, 81.75; H, 5.47; N, 12.75.

3-(3-Nitro-9-carbazolyl)propanenitrile (**6**)

A solution of **5** (5 g, 22 mmol) in nitrobenzene (50 ml) is stirred at room temperature. Fuming nitric acid (2 ml) is added and the mixture is stirred at the same temperature for 1 h. The excess of nitrobenzene is eliminated by steam distillation and the precipitate obtained is filtered, washed and dried to give 5.72 g (98%) of **6** as orange plates; mp: 230-232°C (from isopropanol); ir (KBr): 2240 (CN), 1450 (NO_2) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 3.09 (t, J = 6.32 Hz, 2H, -CH_2CN), 4.84 (t, J = 6.32 Hz, 2H, NCH_2-), 7.35 (t, J = 7.73 Hz, 1H, $H_{arom.}$), 7.83 (d, J = 7.73 Hz, 1H, $H_{arom.}$), 7.92 (d, J = 7.73 Hz, 1H, $H_{arom.}$), 8.35 (dd, $J_{1,2}$ = 8.73 Hz and $J_{2,4}$ = 2.69 Hz, 1H, H_2), 8.40 (d, $J_{1,2}$ = 8.73 Hz, 1H, H_1), 9.16 (d, $J_{2,4}$ = 2.69 Hz, 1H, H_4); ms: (m/z) 266 (M^+). *Anal.* Calcd for $C_{15}H_{11}N_3O_2$: C, 67.92; H, 4.18; N, 15.84. Found C, 67.96; H, 4.15; N, 15.86.

3-(3-Nitro-9-carbazolyl)propanoic acid (**7**)

A solution of sodium hydroxide (2.5N NaOH, 30 ml) is added to a solution of **6** (2 g, 7.5 mmol) in ethanol (30 ml). The mixture is heated at 80°C for 2 h to give a red limpid solution. After cooling, the solution obtained is washed twice with ether. The aqueous phase is then acidified to pH 2-3 with concentrated sulfuric acid. The green precipitate formed is collected by filtration, washed and dried to give 1.92 g (90%) of **8** as a green powder; mp: 240°C (from isopropanol); ir (KBr): 3500-3000 (OH), 1490 (NO_2) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 2.81 (t, J = 6.71 Hz, 2H, -CH_2COOH), 4.70 (t, J = 6.71 Hz, 2H, NCH_2-), 7.31 (t, J = 7.70 Hz, 1H, $H_{arom.}$),

7.58 (t, J = 7.70 Hz, 1H, $H_{arom.}$), 7.76 (d, J = 7.70 Hz, 1H, $H_{arom.}$), 7.83 (d, J = 7.70 Hz, 1H, $H_{arom.}$), 8.34 (dd, $J_{1,2}$ = 8.69 Hz and $J_{2,4}$ = 2.37 Hz, 1H, H_2), 8.40 (d, $J_{1,2}$ = 8.69 Hz, 1H, H_1), 9.16 (d, $J_{2,4}$ = 2.37 Hz, 1H, H_4); ms: (m/z) 285 (M^+). *Anal.* Calcd for $C_{15}H_{12}N_2O_4$: C, 63.38; H, 4.25; N, 9.85. Found C, 63.42; H, 4.22; N, 9.81.

Methyl 3-(3-nitro-9-carbazolyl)propionate (**8a**)

A solution of **7** (0.5 g, 1.7 mmol) in methanol (50 ml) is stirred at 90°C for 4 h with a catalytic amount of concentrated sulfuric acid (5 drops). The solvent is removed by evaporation *in vacuo* and the desired product is purified by column chromatography with 5% methanol in methylene chloride as the eluent. The solvent is evaporated to give 0.415 g (82%) of **8a** as a yellow powder; mp: 108°C (from isopropanol); ir (KBr): 1710 (ester), 1490 (NO_2) cm^{-1}; ^1H nmr ($CDCl_3$): δ = 2.91 (t, J = 6.91 Hz, 2H, -CH_2COOMe), 3.64 (s, 3H, COOCH_3), 4.69 (t, J = 6.91 Hz, 2H, NCH_2-), 7.36 (t, J = 7.30 Hz, 1H, $H_{arom.}$), 7.46-7.63 (m, 3H, $H_{arom.}$), 8.13 (d, $J_{1,2}$ = 8.08 Hz, 1H, H_1), 8.38 (dd, $J_{1,2}$ = 8.08 Hz and $J_{2,4}$ = 1.98 Hz, 1H, H_2), 8.97 (d, $J_{1,4}$ = 1.98 Hz, 1H, H_4); ms: (m/z) 299 (M^+). *Anal.* Calcd for $C_{16}H_{14}N_2O_4$: C, 64.43; H, 4.73; N, 9.39. Found C, 64.40; H, 4.75; N, 9.37.

Benzyl 3-(3-nitro-9-carbazolyl)propionate (**8b**)

A solution mixture of **7** (1 g, 3.52 mmol) and concentrated sulfuric acid (5 drops) in benzyl alcohol (15 ml) is heated at 90°C for 10 h in the presence of benzene (5 ml). After cooling the organic phase is washed twice with a saturated solution of sodium carbonate and the excess of benzyl alcohol is eliminated by evaporation under reduced pressure. The product is purified by column chromatography with gradual ratio of ethyl ether in petroleum ether as the eluent. The solvent is evaporated *in vacuo* to provide 1.15 g (87%) of **8b** as pale yellow plates; mp: 124°C (from isopropanol); ir (KBr): 1710 (ester), 1490 (NO_2) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 2.98 (t, J = 6.71 Hz, 2H, -CH_2COOBn), 4.79 (t, J = 6.71 Hz, 2H, NCH_2-), 4.97 (s, 2H, -COOCH_2Ph), 7.14-7.19 (m, 2H, $H_{arom.}$), 7.25-7.29 (m, 2H, $H_{arom.}$), 7.35 (t, J = 7.90 Hz, 1H, $H_{arom.}$), 7.58 (t, J = 7.90 Hz, 1H, $H_{arom.}$), 7.74 (d, J = 7.90 Hz, 1H, $H_{arom.}$), 7.81 (d, J = 7.90 Hz, 1H, $H_{arom.}$), 8.31 (dd, $J_{1,2}$ = 8.29 Hz and $J_{2,4}$ = 2.37 Hz, 1H, H_2), 8.40 (d, $J_{1,2}$ = 8.29 Hz, 1H, H_1), 9.16 (d, $J_{2,4}$ = 2.37 Hz, 1H, H_4); ms: (m/z) 375 (M^+). *Anal.* Calcd for $C_{22}H_{18}N_2O_4$: C, 70.58; H, 4.84; N, 7.48. Found C, 70.60; H, 4.81; N, 7.49.

Methyl 3-(3-amino-9-carbazolyl)propionate (**9a**)

A mixture of **8a** (0.5 g, 1.6 mmol) and $SnCl_2$ (2 g, 8 mmol) in methanol (10 ml) is refluxed for 4 h. After cooling the amine function is released by addition of 10% sodium hydroxide. The tin salt is eliminated by filtration and the limpid solution obtained is washed twice with methylene chloride. The organic phase is evaporated to dryness and the product is purified by column chromatography with methylene chloride / methanol (95:5) as the eluent to give 0.29 g of **9a** (64%); mp: 108°C (from isopropanol); ir (KBr): 3390 (NH_2), 1710 (ester) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 2.76 (t, J = 6.75 Hz, 2H, -CH_2COOMe), 3.51 (s, 3H, $COOCH_3$), 4.54 (t, J = 6.75 Hz, NCH_2-), 6.53 (dd, $J_{1,2}$ = 8.49 Hz and $J_{2,4}$ = 2.73 Hz, 1H, H_2), 7.07 (t, J = 7.70 Hz, 1H, $H_{arom.}$), 7.24 (d, $J_{2,4}$ = 2.73 Hz, 1H, H_4), 7.27 (d, J = 7.70 Hz, 1H, $H_{arom.}$), 7.48 (d, J = 7.70 Hz, 1H, $H_{arom.}$), 7.91 (d, $J_{1,2}$ = 8.49 Hz, 1H, H_1); ms: (m/z) 269 (M^+). *Anal.* Calcd for $C_{16}H_{16}N_2O_2$: C, 71.63; H, 6.01; N, 10.44. Found C, 71.60; H, 6.03; N, 10.42.

Benzyl 3-(3-amino-9-carbazolyl)propionate (**9b**)

As previously described for **9a**, a mixture of **8b** (1.23 g, 3.30 mmol), $SnCl_2$ (5.37 g, 18.3 mmol) in ethyl acetate (15 ml) is refluxed for 10 h. The cooled solution is treated as for the methyl ester (**9a**) and the product is purified by column chromatography with gradual ratio of ethyl ether in petroleum ether as the the eluent. The solvent is eliminated by evaporation *in vacuo* to provide 0.83 g of **9b** (82%) as pale brown plates; mp: 80°C (from isopropanol); ir (KBr): 3390 (NH_2), 1710 (ester) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 2.82 (t, J = 6.71 Hz, 2H, -CH_2COOBn), 4.56 (t, J = 6.71 Hz, 2H, NCH_2-), 4.75 (s, 2H, NH_2), 4.97 (s, 2H, -CH_2Ph), 6.80 (dd, $J_{1,2}$ = 8.29 Hz and $J_{2,4}$ = 2.37 Hz, 1H, H_2), 7.07 (t, J = 7.70 Hz, 1H, $H_{arom.}$), 7.17-7.22 (m, 2H, $H_{arom.}$), 7.26-7.36 (m, 5H, $H_{arom.}$), 7.36 (d, J = 7.70 Hz, 1H, $H_{arom.}$), 7.92 (d, J = 8.29 Hz, 1H, H_1); ms: (m/z) 345 (M^+). *Anal.* Calcd for $C_{22}H_{20}N_2O_2$: C, 76.72; H, 5.85; N, 8.13. Found C, 76.70; H, 5.82; N, 8.15.

Benzyl 3-(3-acetamido-9-carbazolyl)propionate (**10b**)

A solution of **9b** (0.08 g, 0.23 mmol) and acetic anhydride (0.65 ml, 0.69 mmol) in pyridine (2 ml) is stirred at room temperature for 10 h. To the solution obtained, methanol (1 ml) is added at 0°C to hydrolyze the excess acetic anhydride. After 15 min of stirring, the solvent is evaporated *in vacuo*. The pale brown product obtained is then washed with methylene chloride and dried to provide 0.98 g of **10b** (99%); mp 158°C (from isopropanol); ir (KBr): 3390 (NH), 1710 (ester) cm^{-1}; ^1H nmr ($CDCl_3$): δ = 2.26 (s, 3H, $COCH_3$), 2.92 (t, J = 7.11 Hz, 2H, -CH_2COOBn), 4.65 (t, J = 7.11 Hz, 2H, NCH_2-), 5.08 (s, 2H, -CH_2Ph), 7.20-7.50 (m, 10H,

$H_{arom.}$), 8.06 (d, $J_{1,2}$ = 7.9 Hz, 1H, H_1), 8.31 (d, $J_{2,4}$ = 1.18 Hz, 1H, H_4); ms: (m/z) 387 (M^+). *Anal.* Calcd for $C_{24}H_{22}N_2O_3$: C, 74.60; H, 5.74; N, 7.25. Found C, 74.63; H, 5.70; N, 7.22.

Benzyl 3-(N-tBoc-L-alanine-3-amido-9-carbazolyl)propionate (**10c**)

A solution of terbutoxycarbonyl-L-Alanine (0.027 g, 0.15 mmol) in dimethylformamide (4 ml) is stirred at 0°C in the presence of hydroxybenzotriazole (HOBt, 0.02 g, 0.15 mmol) and dicyclohexylcarbodiimide (DCC, 0.033 g, 0.15 mmol). After stirring for 5 min, **9b** (0.05 g, 0.15 mmol) is added and the mixture is stirred at 0°C for 2 h and at room temperature for 10 h. The white precipitate of dicyclohexylurea is eliminated by filtration, the solvent is evaporated *in vacuo* and the product is purified by column chromatography with ethyl acetate / cyclohexane (5:5) as the eluent. The solvent is evaporated to provide 0.072 g (93%) of **10c** as a vitrous colorless syrup; ir (film): 3500-3000 (COOH), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 1.29 (d, J = 7.11 Hz, 3H, CH-CH_3), 1.39 (s, 9H, -$C(CH_3)_3$), 2.89 (t, J = 6.64 Hz, 2H, -CH_2COOBn), 4.16 (t, J = 6.87 Hz, CH_α (Ala)), 4.65 (t, J = 6.64 Hz, 2H, NCH_2-), 4.96 (s, 2H, -CH_2Ph), 7.00 (d, J = 7.19 Hz, 1H, tBOC-NH-), 7.14-7.31 (m, 7H, $H_{arom.}$), 7.41 (t, J = 7.19 Hz, 1H, $H_{arom.}$), 7.41-7.54 (m, 2H, $H_{arom.}$), 8.04 (d, $J_{1,2}$ = 7.70 Hz, 1H, H_1), 8.40 (s, 1H, H_4), 9.89 (s, 1H, $NH_{arom.}$); ms: (m/z) 516 (M^+). *Anal.* Calcd for $C_{30}H_{33}N_3O_5$: C, 69.99; H, 6.45; N, 8.15. Found C, 69.95; H, 6.42; N, 8.17.

3-(3-Acetamido-9-carbazolyl)propionic acid (**11a**)

A solution of **10a** (0.176 g, 0.45 mmol) in dimethylformamide (4 ml) is stirred in a low pressure of hydrogen in the presence of 10% of Pd/C (0.01 g). After stirring for 4 h, the solution is filtered on celite, the solvent is eliminated by evaporation and the product is purified by column chromatography with methylene chloride / methanol (9:1) as the eluent. The solvent is evaporated to dryness to give 0.12 g (90%) of **11a** as a vitrous sirup; ir (KBr): 3500-3000 (COOH), 1700 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 2.09 (s, 3H, CH_3CO-), 2.66 (t, J = 6.8 Hz, 2H, -CH_2COOH), 4.58 (t, J = 6.9 Hz, 2H, NCH_2-), 7.16 (t, J = 7.2 Hz, 1H, $H_{arom.}$), 7.43 (t, J = 7.2 Hz, 1H, $H_{arom.}$), 7.52 - 7.63 (m, 2H, $H_{arom.}$), 8.02 (d, $J_{1,2}$ = 7.2 Hz, 1H, H_1) 8.36 (s, 1H, H_4), 9.94 (s, 1H, $NH_{arom.}$); ms: (m/z) 297 (M^+). *Anal.* Calcd for $C_{17}H_{16}N_2O_3$: C, 68.91; H, 5.44; N, 9.45. Found C, 68.93; H, 5.43; N, 9.42.

3-(N-tBoc-L-alanine-3-amido-9-carbazolyl)propionic acid (**11b**)

A solution of **10b** (0.176 g, 0.34 mmol) in dimethylformamide (3 ml) is stirred in a low pressure of hydrogen in presence of Pd/C (10% Pd, 0.05 g). The hydrogenolysis occurs during 4 h and the solution is filtered on celite. The solvent is eliminated by evaporation and the product is purified by column chromatography with ethyl ether / petrol ether (3:7) as the eluent. The solvent is removed by evaporation *in vacuo* to provide 0.131 g (90%) of **11b** as a vitrous colorless sirup; ir (film): 3500-3000 (COOH) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 1.28 (d, J = 7.11 Hz, 3H, CH_α-CH_3), 1.38 (s, 9H, -C(CH_3)$_3$), 2.71 (t, J = 6.91 Hz, 2H, -CH_2COOH), 4.16 (t, J = 6.91 Hz, 1H, CH_α (Ala)), 4.59 (t, J = 6.91 Hz, 2H, NCH_2-), 6.98 (d, J = 6.64 Hz, 1H, tBOC-NH-), 7.17 (t, J = 7.58 Hz, $H_{arom.}$), 7.42 (t, J = 7.58 Hz, 1H, $H_{arom.}$), 7.55-7.59 (m, 2H, $H_{arom.}$), 8.04 (d, $J_{1,2}$ = 7.58 Hz, 1H, H_1), 8.37 (s, 1H, H_4), 9.87 (s, 1H, $NH_{arom.}$); ms (m/z) 426 (M^+). *Anal.* Calcd for $C_{23}H_{27}N_3O_5$: C, 64.93; H, 6.40; N, 9.87. Found C, 64.95; H, 6.38; N, 9.90.

Methyl 7-ethoxycarbonylaminocoumarin-4-acetate (**13a**)

A mixture of *m*-ethoxycarbonylaminophenol (**12**) (1 g, 5.52 mmol) and dimethylacetonedicarboxylate (0.9 ml, 5.52 mmol), is heated with stirring for 10 min at 90°C. Sulfuric acid (0.1 ml) is added and the yellow mixture is stirred for 30 min at 90°C to give a pale yellow mass. After cooling, the colorless residual solid is dissolved in methylene chloride and the product is purified by flash chromatography on a column of silica gel 60 (230-400 mesh) with cyclohexan / ethyl acetate (7:3) as the eluent. The solvent is removed by evaporation to give 1.1 g (66%) of **13a** as colorless cubes; mp: 174°C (from ethanol); ir (KBr): 3280 (NH), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 1.26 (t, J = 7.11 Hz, 3H, CH_2-CH_3), 3.64 (s, 3H, $COOCH_3$), 3.96 (s, 2H, -CH_2COOMe), 4.18 (q, J = 7.11 Hz, 2H, CH_2-CH_3), 6.33 (s, 1H, H_3), 7.38 (dd, $J_{5,6}$ = 9.08 Hz and $J_{6,8}$ = 2.37 Hz, 1H, H_6), 7.55 (d, $J_{6,8}$ = 2.37 Hz, 1H, H_8), 7.62 (d, $J_{5,6}$ = 9.08 Hz, 1H, H_5), 10.13 (s, 1H, EtCONH); ms: (m/z) 306 (M^+). *Anal.* Calcd for $C_{15}H_{15}NO_6$: C, 59.03; H, 4.94; N, 4.59. Found C, 59.05; H, 4.90; N, 4.56.

Ethyl 7-ethoxycarbonylaminocoumarin-4-acetate (**13b**)

As described for the methyl ester, **12** (2.36 g, 13.0 mmol), diethylacetonedicarboxylate (2.1 ml, 14.3 mmol) are heated at 90°C in the presence of traces of H_2SO_4 (0.1 ml). A mixture of **13b** (2.69 g, 65%) and **14** (7%) is obtained (in accordance with the nmr and hplc analysis). The colorless mass can be dissolved in hot ethanol and after cooling the white precipitate formed is purified by filtration, washed and dried to provide 2 g (49%) of pure **13b**; mp: 180°C (from ethanol); ir (KBr): 3290 (NH), 1710 (C=O) cm^{-1};^1H nmr (DMSO-d_6): δ = 1.18 (t,

J = 7.11 Hz, 3H, $COOCH_2CH_3$), 1.26 (t, J = 7.11 Hz, 3H, OCH2CH_3), 3.95 (s, 2H, -CH_2COOEt), 4.1 (q, J = 7.11 Hz, 2H, $COOCH_2CH_3$), 4.19 (q, J = 7.11 Hz, 2H, OCH_2CH_3), 6.34 (s, 1H, H_3), 7.39 (dd, $J_{5,6}$ = 8.90 Hz and $J_{6,8}$ = 1.80 Hz, H, H_6), 7.56 (d, $J_{6,8}$ = 1.80 Hz, 1H, H_8), 7.62 (d, $J_{5,6}$ = 8.90 Hz, 1H, H_5), 10.14 (s, 1H, CONH); ms: (m/z) 320 (M^+). *Anal.* Calcd for $C_{16}H_{17}NO_6$: C, 60.19; H, 5.37; N, 4.39. Found C, 60.22; H, 5.34; N, 4.36.

7-Ethoxycarbonylamino-4-methylcoumarin (**14**)

This compound is obtained as a secondary product in the synthesis of **13c** and **13b** with a yield of 5 and 7%; mp: 188°C (from ethanol); ir (KBr)): 3290 (NH), 1710 (C=O) cm^{-1}, ^1H nmr (DMSO-d_6): δ = 1.26 (t, J = 7.11 Hz, 3H, OCH_2CH_3), 2.37 (s, 3H, CH_3), 4.16 (q, J = 7.11 Hz, 2H, OCH_2CH_3), 6.21 (s, 1H, H3), 7.41 (dd, $J_{5,6}$ = 8.69 Hz and $J_{6,8}$ = 1.97 Hz, 1H, H_6), 7.54 (d, $J_{6,8}$ = 1.97 Hz, 1H, H_8), 7.66 (d, $J_{5,6}$ = 8.69 Hz, 1H, H_5), 10.09 (s, 1H, CONH); ms (m/z) 248 (M^+). *Anal.* Calcd for $C_{13}H_{13}NO_4$: C, 63.16; H, 5.30; N, 5.66. Found C, 63.18; H, 5.28; N, 5.70.

7-Aminocoumarin-4-acetic acid (**15**)

A mixture of **13a** (0.5 g, 1.56 mmol) and NaOH (0.624 g, 15.6 mmol) in water (10 ml) is heated under reflux for 12 h. After cooling, concentrated sulfuric acid is added until a brown precipitate is formed. Water is removed by evaporation and the product is purified by column chromatography with gradual ratio of methanol in methylene chloride as the eluent. The solvent is evaporated to give 0.27 g (78%) of **15** as orange cubes; mp: 255°C (decomp.) (from ethanol); ir (KBr): 3440-3380 (NH_2), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 3.52 (s, 2H, -CH_2COOH), 5.97 (s, 1H, H_3), 6.41 (d, $J_{6,8}$ = 2.27 Hz, 1H, H_8), 6.54 (dd, $J_{5,6}$ = 8.69 Hz and $J_{6,8}$ = 2.27 Hz, 1H, H_6), 7.23 (d, $J_{5,6}$ = 8.69 Hz, 1H, H_5); ms (m/z) 220 (M^+). *Anal.* Calcd for $C_{11}H_9NO_4$: C, 60.28; H, 4.14; N, 3.39. Found C, 60.30; H, 4.10; N, 3.40.

Methyl 7-aminocoumarin-4-acetate (**16a**)

A solution of **15** (0.05 g, 0.228 mmol), 4-dimethylaminopyridine (DMAP, 0.014 g, 0.114 mmol) in methanol (2 ml) is cooled by stirring in an ice bath. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDCI, 0.07 g, 0.25 mol) is added and the reaction mixture is stirred at 0°C for 2 h and at room temperature for 10 h. The solvent is removed by evaporation *in vacuo* and the product is purified by column chromatography with methylene chloride / methanol (95:5) as the eluent. The solvent is evaporated to dryness to provide 0.044 g

of **16a** (83%) as a yellow powder; mp: 180°C (from ethanol); ir (KBr): 3440-3340 (NH_2), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d6): δ = 3.62 (s, 3H, $COOCH_3$), 3.84 (s, 2H, -CH_2COOMe), 5.98 (s, 1H, H_3), 6.15 (s, 2H, NH_2), 6.41 (d, $J_{6,8}$ = 2.11 Hz, 1H, H_8), 6.54 (dd, $J_{5,6}$ = 8.81 Hz and $J_{6,8}$ = 2.11 Hz, 1H, H_6), 7.22 (d, $J_{5,6}$ = 8.81 Hz, 1H, H_5); ms: (m/z) 234 (M^+). *Anal.* Calcd for $C_{12}H_{11}NO_4$: C, 61.81; H, 4.75; N, 6.01. Found C, 61.83; H, 4.73; N, 6.03.

Benzyl 7-aminocoumarin-4-acetate (**16b**)

As described for **16a**, **15** (0.22 g, 1 mmol) is stirred in the presence of DMAP (0.062 g, 0.5 mmol), EDCI (0.262 g, 1.1 mmol), in benzyl alcohol (5 ml). The product is purified by the method used for **16a** to give 0.25 g (81%) of **16b** as a yellow powder; mp: 187-188°C (from ethanol); ir (KBr): 3440-3340 (NH_2), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 3.91 (s, 2H, -CH_2COOBn), 5.14 (s, 2H, -COOCH_2Ph), 6.01 (s, 1H, H_3), 6.16 (s, 2H, NH_2), 6.43 (d, $J_{6,8}$ = 1.93 Hz, 1H, H_8), 6.52 (dd, $J_{5,6}$ = 8.85 Hz and $J_{6,8}$ = 1.93 Hz, 1H, H_6), 7.28-7.43 (m, 6H, $H_{arom.}$); ms (m/z) 310 (M^+). *Anal.* Calcd for $C_{18}H_{15}NO_4$: C, 69.90; H, 4.89; N, 4.53. Found C, 69.92; H, 4.86; N, 4.56.

Benzyl 7-aminocoumarin-4-acetamidoacetate (**17a**)

A solution of **15** (0.1 g, 4.57 mmol) in dimethylformamide (5 ml) is stirred at 0°C in the presence of HOBt (0.08 g, 5.03 mmol) and DCC (0.104 g, 5.03 mmol). After stirring for 5 min, glycine benzyl ester-*p*-tosylate (1.54 g, 5.03 mmol) and triethylamine (0.7 ml, 5.03 mmol) are added. The mixture is stirred at 0°C for 2 h and at room temperature for 10 h. The white precipitate of dicyclohexylurea is eliminated by filtration and the solvent is evaporated to dryness. The mass obtained is dissolved in methylene chloride and the product is purified by column chromatography with methylene chloride / methanol (95:5) as the eluent. The solvent is removed *in vacuo* to give 0.132 g (79%) of **17a** as a pale yellow powder; mp: 250°C (from ethanol); ir (KBr): 3440-3340 (NH_2), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 3.63 (s, 2H, CH_2CONH), 3.94 (d, J = 5.92 Hz, 2H, NHCH_2(Gly)), 5.12 (s, 2H, COOCH_2Ph), 5.97 (s, 1H, H_3), 6.10 (s, 2H, NH_2), 6.41 (d, $J_{6,8}$ = 1.76 Hz, 1H, H_8), 6.53 (dd, $J_{5,6}$ = 8.69 Hz and $J_{6,8}$ = 1.76 Hz, 1H, H_6), 7.30-7.40 (m, 5H, $H_{arom.}$), 7.43 (d, $J_{5,6}$ = 8.69 Hz, 1H, H_5), 8.64 (t, J = 5.92 Hz, 1H, CONH(Gly)). ms: (m/z) 367 (M^+). *Anal.* Calcd for $C_{20}H_{18}N_2O_5$: C, 65.57; H, 4.95; N, 7.64. Found C, 65.58; H, 4.92; N, 7.66.

7-Aminocoumarin-4-acetic acid hexylamide (**17b**)

As described for **16b**, **15** (0.5 g, 2.28 mmol) in dimethylformamide (4 ml) is stirred for 2 h at 0°C and 10 h at room temperature in the presence of HOBt (0.34 g, 2.51 mmol), DCC (0.52 g, 2.51 mmol) and hexylamine (0.3 ml, 2.51 mmol). The solution is treated the same way as for **17a** and the product is purified by the same column chromatographical system to give 0.531 g (78%) of **17b** as a pale yellow powder; mp: 195°C (from ethanol); ir (KBr) : 3440-3340 (NH_2), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 0.81-0.94 (m, 3H, hexylamine), 1.18-1.44 (m, 8H, hexylamine), 3.04 (q, J = 5.92 Hz, 2H, CONH-CH_2), 3.53 (s, 2H, CH_2-CONH), 5.92 (s, 1H, H_3), 6.10 (s, 2H, NH_2), 6.41 (d, $J_{6,8}$ = 2.21 Hz, 1H, H_8), 6.53 (dd, $J_{5,6}$ = 8.53 Hz and $J_{6,8}$ = 2.21 Hz, 1H, H_8), 7.42 (d, $J_{5,6}$ = 8.53 Hz, 1H, H_5), 8.10 (t, J = 5.92 Hz, 1H, CONH); ms: (m/z) 303 (M^+). *Anal.* Calcd for $C_{17}H_{22}N_2O_3$: C, 67.53; H, 7.33; N, 9.26. Found C, 67.55; H, 7.32; N, 9.24.

Methyl 7-acetamidocoumarin-4-acetate (**18a**)

A solution of **16a** (0.044 g, 0.118 mmol) and acetic anhydride (1 ml, 0.940 mmol) in pyridine (3 ml) is stirred for 10 h at room temperature. To the solution obtained, methanol (1 ml) is added at 0°C to hydrolyze the excess acetic anhydride. The white precipitate formed is filtered, washed with 10% methanol in methylene chloride and dried to provide 0.042 g (81%) of **18a** as a white powder; mp: 258-260 °C (from ethanol); ir (KBr): 3290 (NH), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-6): δ = 2.10 (s, 3H, NHCOCH_3), 3.64 (s, 3H, COOCH_3), 3.88 (s, 2H, CH_2COOMe), 6.37 (s, 1H, H_3), 7.43 (dd, $J_{5,6}$ = 8.69 Hz and $J_{6,8}$ = 1.60 Hz, H, H_6), 7.12 (d, $J_{5,6}$ = 8.69 Hz, 1H, H_5), 7.78 (d, $J_{6,8}$ = 1.60 Hz, 1H, H_8), 10.38 (s, 1H, MeCONH); ms: (m/z) 276 (M^+). *Anal.* Calcd for $C_{14}H_{13}NO_5$: C, 61.10; H, 4.76; N, 5.09. Found C, 61.12; H, 4.78; N, 5.07.

Benzyl 7-acetamidocoumarin-4-acetate (**18b**)

Using the same procedure described for **18a**, **16b** (0.05 g, 0.162 mmol) is treated by acetic anhydride (0.76 ml, 0.81 mmol) in the presence of pyridine (3 ml) at room temperature for 10 h. After addition of methanol (1 ml), the product is purified by column chromatography with methylene chloride as the eluent. The solvent is evaporated to give 0.04 g (71%) of **18b** as a white powder; mp: 198°C (from ethanol); ir (KBr): 3290 (NH), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 2.08 (s, 3H, NHCOCH_3), 4.04 (s, 2H, CH_2COOBn), 5.15 (s, 2H, COOCH_2Ph), 6.36 (s, 1H, H_3), 7.25-7.48 (m, 5H, $H_{arom.}$), 7.59 (dd, $J_{5,6}$ = 8.69 Hz and $J_{6,8}$ = 1.67 Hz, 1H, H_6), 7.69 (d, $J_{5,6}$ = 8.69 Hz, 1H, H_5), 7.77 (d, $J_{6,8}$ = 1.67 Hz, 1H, H_8), 10.36 (s, 1H, MeCONH); ms (m/z) 352 (M^+). *Anal.* Calcd for $C_{20}H_{17}NO_5$: C, 68.38; H, 4.87; N, 3.98. Found C, 68.40; H, 4.86; N, 3.99.

Benzyl 7-acetamidocoumarin-4-acetamidoacetate (**19**)

Following the same procedure described for **18a**, **17a** (0.045 g, 0.122 mmol) is treated by acetic anhydride (1 ml, 0.940 mmol) in the presence of pyridine (3 ml) at room temperature for 10 h. The purification of the product is the same as for **18a** and provides 0.033 g (66%) of **19** as a white powder; mp: 249-250°C (from ethanol); ir (KBr): 3300 (NH), 1710 (C=O) cm^{-1}; ^1H nmr (DMSO-d_6): δ = 2.08 (s, 3H, $COCH_3$), 3.75 (s, 2H, -CH_2CONH), 3.95 (d, J = 5.92 Hz, 2H, $NHCH_2$(Gly)), 5.11 (s, 2H, $COOCH_2Ph$), 6.32 (s, 1H, H_3), 7.29-7.39 (m, 5H, $H_{arom.}$), 7.42 (dd, $J_{5,6}$ = 8.69 Hz and $J_{6,8}$ = 1.58 Hz, 1H, H_6), 7.70 (d, $J_{5,6}$ = 8.69 Hz, 1H, H_5), 7.75 (d, $J_{6,8}$ = 1.58 Hz, 1H, H_8), 8.69 (t, J = 5.92 Hz, 1H, CONH(Gly)), 10.35 (s, 1H, MeCONH); ms (m/z) 409 (M^+). *Anal.* Calcd for $C_{22}H_{20}N_2O_6$: C, 64.73; H, 4.90; N, 6.86. Found C, 64.75; H, 4.88; N, 6.87.

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